

Chilling Injury and Nucleotide Changes in Young Cotton Plants¹

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ABSTRACT

The effects of chilling at 3 to 5 C on the nucleotide composition of roots and leaves of cotton (*Gossypium hirsutum* L.) seedlings were determined. Chilling decreased the concentration of nucleotides, especially di- and triphosphates, in both leaves and roots. Chilling also caused an increase in free nucleosides. The results are interpreted to mean that general phosphorolytic activity is associated with chilling injury rather than damage to the phosphorylating mechanisms alone. Hardening at 10 to 20 C prior to chilling protected the seedlings against subsequent chilling injury and prevented nucleotide losses.

We reported earlier (14) that chilling markedly decreased ATP, especially in leaves, when young unhardened cotton plants were chilled. Hardening at 15 C prior to chilling at 5 C prevented the decreases in ATP and caused the plants to be much more resistant to chilling injury. Decreases in ATP could result from decreased rates of oxidative- and photo-phosphorylation in mitochondria (10) and chloroplasts, respectively, from increased phosphorolytic activity, or both. In the first case an increase in mono- and diphosphates would be expected, whereas in the other cases, a decrease in phosphorylation at all levels would be expected. In this paper we report the effects of chilling temperature on the major nucleotides extracted from young cotton leaves and roots. The influence of hardening upon these effects is also reported.

MATERIALS AND METHODS

Cotton seedlings (*Gossypium hirsutum* L., cv. Parrott or Westburn) were cultured for 2 to 3 weeks as previously described (15), then subjected to hardening or chilling temperatures or both for 2 days each in climate-control chambers at about 3,000 ft-c during 14-hr illumination each day. Hardening temperatures were 15 C both day and night in some experiments and were 20 C day and 10 C night in others. Chilling temperatures were 3 to 5 C. Four treatments were used: control or unhardened-not chilled, unhardened-chilled, hardened-not chilled, and hardened-chilled. Roots and leaves were har-

vested, rinsed successively in tap and distilled water (roots only), frozen, lyophilized, ground to pass a 60-mesh screen, and stored over CaCl₂ at -26 C until assayed.

Portions of powdered leaf tissue, 6.0 g each, were homogenized for 5 min at 140 v in the 200-ml cup of a Sorvall⁴ Omni-mixer with 120 ml of 5% (w/v) trichloroacetic acid while the cup was cooled in ice-water. In some tests 30 ml of cold chloroform were added to remove lipids. The homogenate was centrifuged at 27,000g for 10 min at 0 C, and the upper (aqueous) fraction was retained. In addition to nucleotides, this fraction contained anthocyanin and phenolic substances which interfered with chromatographic separation and identification of nucleotides. Consequently, 100-ml portions of the extract were extracted four times with 20-ml portions of cold *n*-amyl alcohol (13). (We subsequently discovered that passing the extract through Sephadex G-10 was effective in removing these interfering materials.) Phases were separated by centrifugation after each extraction. The aqueous phase was then extracted twice with 40-ml portions of ethyl ether to remove residual trichloroacetic acid and *n*-amyl alcohol. All operations were conducted at 4 C or less. Residual ether was removed *in vacuo*, the pH was adjusted to 7.1 with KOH, and the resulting suspension was centrifuged to remove a fine precipitate prior to chromatographic fractionation.

The acid extracts from root tissue contained less interfering material than those from leaves. Therefore, root extracts were not treated with *n*-amyl alcohol. Because of the limited amount of root tissue, less than 6 g were used in some cases. Otherwise, extraction of nucleotides from roots was similar to the procedure outlined above for leaf tissue.

Dowex 1 × 8 anion exchange resin (Cl⁻ form, 200-400 mesh) was graded, cleaned, and converted to the formate form as outlined by Cohn (5). Resin columns 1.2 × 50 cm were used for fractionating leaf nucleotides and, in some cases, 0.8- × 60-cm columns were used for fractionating root nucleotides. The method of Cherry and Hageman (3) was used and 4.6-ml fractions were collected at flow rates of 0.8 to 1.0 ml/min. Absorption at 260 nm was determined for each fraction, and elution chromatograms were prepared for each treatment by plotting absorbance versus fraction number.

Tentative identification of nucleotides was made by comparing relative positions and order of elution from the column with those given in published reports (1-3, 7, 8, 16) and with standard mixtures fractionated by the same procedure. For confirmation of peak identity, fractions common to a peak were pooled, then either lyophilized or purified on acid-washed activated charcoal (6). After lyophilization, the residues were dissolved in small volumes of water, and the nucleotides were

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identified by their characteristic absorption spectra in acid, neutral, and basic solutions (12). Interfering material contributing to the background absorbance prevented direct identification of some of the spectra. These samples were further purified by fractionation on columns of Sephadex G-10. Identities were also confirmed by co-chromatography with known standards on thin layer plates of MN-cellulose 300 G with Pabst solvents I and III (12). NAD and NADP were identified by the cyanide addition reaction (4). ATP was identified by light emission with firefly luciferase (15).

All tests were repeated at least twice. Within an experiment, percentage recovery of nucleotide was assumed to be the same for each treatment so no correction was made.

Two experiments were conducted to determine the nucleoside changes with chilling. Two grams of leaf tissue were homogenized with 20 ml of chloroform plus 50 ml of cold 10% (w/v) trichloroacetic acid. After phase separation by centrifugation, the aqueous phase was retained and trichloroacetic acid removed by ether extraction. Many ultraviolet-absorbing impurities were removed by passing the solution through a 2- × 10-cm column of Sephadex G-10. The effluent was adjusted to pH 7.0 with NaOH and lyophilized. The residue was dissolved in a small portion of water, neutralized, and anionic material was removed on a 2- × 5-cm column of Dowex 1 × 8, Cl-form, 200 to 400 mesh. Nucleosides were eluted with a water wash, and the effluent was lyophilized. The residue was dissolved in 3 ml of water and nucleosides fractionated on a

3.8- × 25-cm column of Sephadex G-10 which was preconditioned with 0.01 M formic acid. The column was developed with water and nucleosides eluted in the order cytidine, uridine, guanosine, and adenosine.

RESULTS AND DISCUSSION

The elution patterns of the leaf and root nucleotides, shown in Figures 1 and 2 respectively, were similar to those reported by others for the formate system (1, 2, 7). Identity of the nucleotides in the numbered peaks are listed in Table I. Estimates of the amount of nucleotide in each of the major peaks were possible except for NAD and CMP which were eluted together with an unidentified interfering substance. Some of the peaks contained nonnucleotide compounds which were not identified. Peaks 6 and 7 of the leaf chromatograms were not the same as the correspondingly numbered peaks of the root chromatogram. With the exception of peak 7, which had an adenine-type spectrum, these substances did not appear to be nucleotides. The substance in peak 6 of the leaf chromatogram was present in high concentration in control leaves but was almost totally absent when unhardened leaves were chilled. Hardening prevented loss of this substance during subsequent chilling. In solutions of acid and neutral pH this substance exhibited a minimum absorbance at 220 nm and a maximum at 243 nm. In alkaline solution the point of maximum absorbance shifted to 300 nm. The compound did not contain phosphorus.

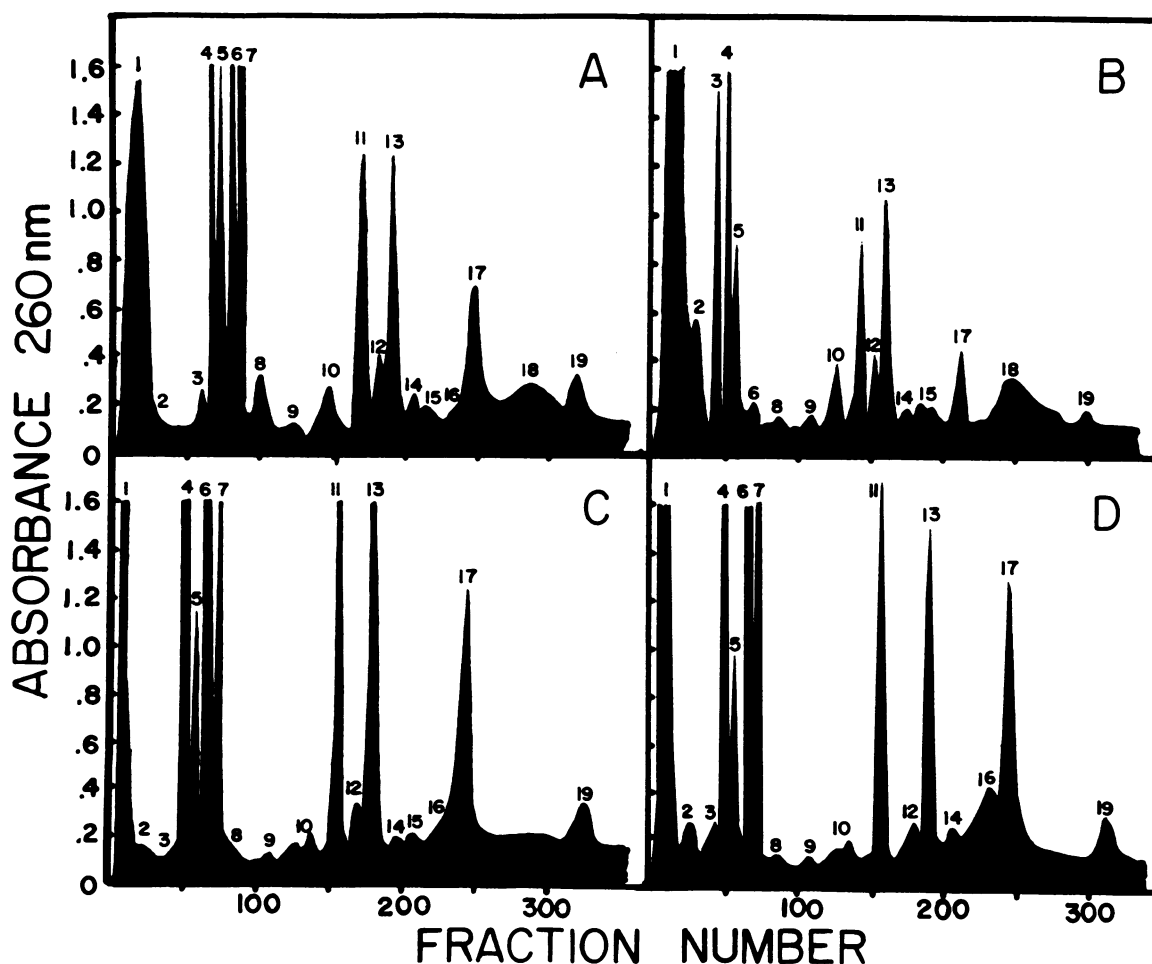


FIG. 1. Comparison of chromatograms of cotton leaf nucleotides from 0.8- × 60-cm columns of Dowex 1 × 8 formate anion exchange resin. Fraction volume was 4.6 ml. A: Control; B: unhardened chilled; C: hardened; D: hardened chilled. Note the general decrease of nucleotides in the unhardened chilled treatment but not in the hardened chilled treatment compared to the control.

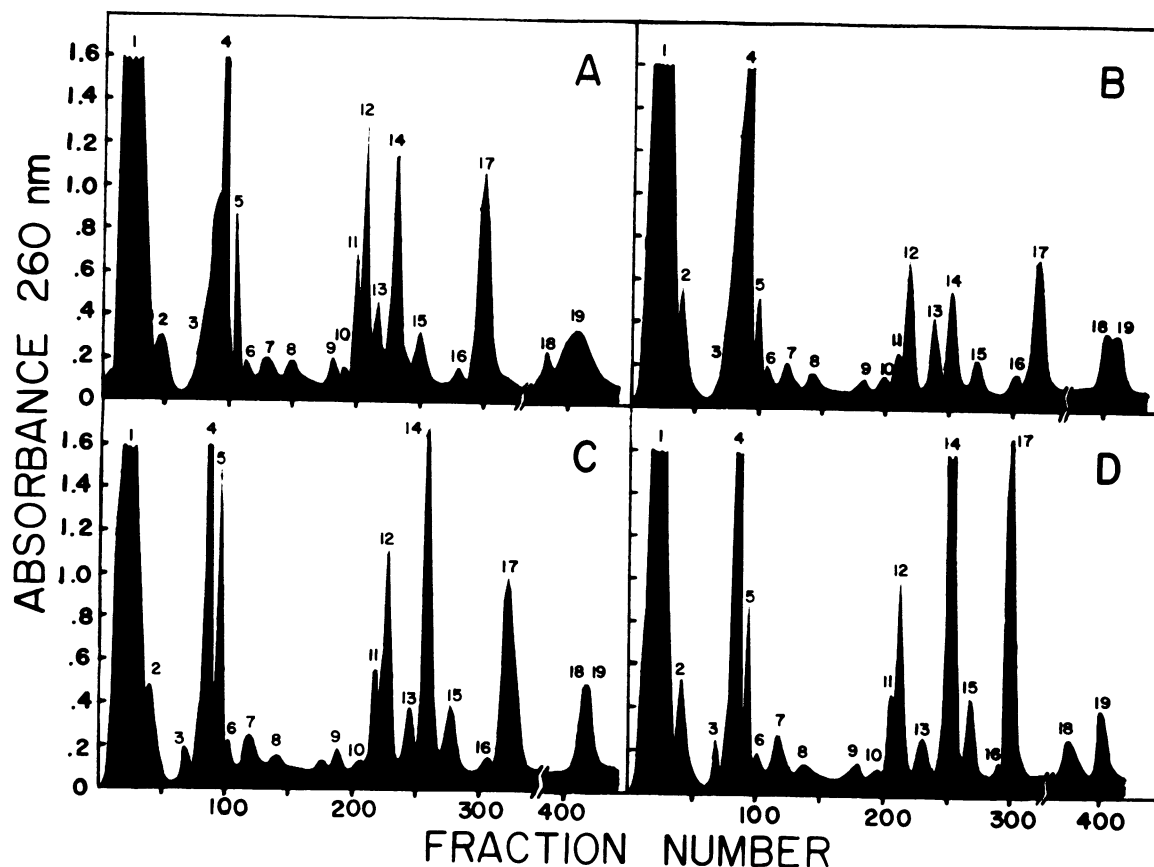


FIG. 2. Comparison of chromatograms of cotton root nucleotides from 1.2×50 -cm columns of Dowex 1×8 formate anion exchange resin. Fraction volume was 4.6 ml. A: Control; B: unhardened chilled; C: hardened; D: hardened chilled. The trend is the same as in leaf tissue.

Table I. Identification of 260-nm Peaks Obtained by Dowex 1×8 Formate Column Chromatography of Cotton Nucleotides

Peak Number	Root	Leaf
1	Unadsorbed	Unadsorbed
2	Unadsorbed	Unadsorbed
3	Mixture	Mixture
4	NAD, CMP	NAD, CMP
5	AMP	AMP
6	Nonnucleotide	Nonnucleotide
7	Adenosine derived	Nonnucleotide
8	NADP	NADP
9	GMP	GMP
10	CDP	UMP
11	UMP	ADP
12	ADP	UDP derived
13	UDP derived	UDP, UDP-hexose
14	UDP, UDP-hexose	NADPH degraded
15	NADPH degraded	Nonnucleotide
16	CTP	CTP
17	ATP	ATP
18	GTP	Nonnucleotide
19	UTP	UTP, GTP

The uridine derivative of peaks 12 and 13 of leaves and roots respectively was not identified; however, peaks 13 and 14 of leaves and roots respectively contained a mixture of UDP and UDP-hexose. For estimation of the effects of chilling, all of these uridine derivatives were pooled. Peaks 14 and

15 of leaves and roots respectively was tentatively identified as the acid decomposition product of NADPH on the bases of its absorbance spectrum and elution position (1). The amount of substance in this peak was taken as a measure of NADPH present.

The effect of chilling on cotton leaf tissue is illustrated in the comparison of Figures 1A (control) and 1B (chilled). Table II gives an estimation of some of the nucleotide changes in three separate experiments. There was a general decline in total leaf nucleotides as well as some non-nucleotides (peaks 6 and 7) with chilling. One exception occurred with the increase of AMP when the plants were chilled in experiment 2. An increase in peak 3 was very apparent in the chilled treatment of all three experiments. This peak contained a mixture of compounds including what appeared to be tryptophan and adenosine. Figures 1B and 1C show a comparison of hardened and hardened-chilled leaf tissue nucleotides. Generally, an increase in nucleotides occurs with hardening, and the hardening process prevents a subsequent loss of nucleotides when the tissue is chilled. The decrease in AMP when hardened plants are chilled may be the result of an increase in ADP and ATP.

The balance of NADP and NADPH may have some importance in the hardening phenomenon as reported by Kuraishi *et al.* (9). Chilling caused a decrease in both NADP and NADPH, whereas the trend in hardening was an increase in NADPH with a corresponding decrease in NADP. Subsequent chilling resulted in only a slight decrease in NADPH.

Figure 2 shows the comparison of the four treatments on root tissue. Again, in one experiment AMP increased with chilling (Table II). Other trends were similar to that observed for leaf tissue except for the changes in ATP. In one experi-

Table II. *Effects of Chilling on the Major Nucleotides in Cotton Tissue*

Nucleotide	Experiment No. ¹	Leaves				Roots			
		UN ²	UC	HN	HC	UN	UC	HN	HC
		<i>μmoles/g dry weight</i>							
AMP	1	379	132	47	154
	2	324	452	431	324	273	150	574	310
	3	291	121	332	305	167	133	209	173
UMP	1	161	53	144	93
	2	131	92	57	58	39	21	42	28
	3	118	57	62	53	56	27	48	49
ADP	1	453	228	282	117
	2	487	256	524	556	686	387	812	622
	3	376	182	440	467	466	198	521	530
UDP derived	1	1004	443	1013	273
	2	887	505	917	691	1646	848	2215	2363
	3	1024	704	1382	1431	1503	523	1736	1777
ATP	1	496	117	600	18
	2	484	128	610	621	1125	739	1177	1212
	3	351	71	379	382	821	319	936	873
UTP	1	138	22	534	91
	2	137	34	164	135	1071	730	725	817
	3	223	69	236	241	424	121	436	420
NADP	1	123	65	79	26
	2	32	23	27	29	82	77	58	58
	3	55	36	38	42	73	35	50	56
NADPH	1	68	32	51	22
	2	43	18	49	43	127	73	167	137
	3	48	27	60	55	109	61	135	122

¹ Experiment 1 of leaves represent different plants from experiment 1 of roots.

² UN: Control or unhardened-not chilled; UC: unhardened-chilled; HN: hardened-not chilled; HC: hardened-chilled.

ment a very significant increase in ATP occurred with the chilling of hardened tissue, while in the other a small decrease occurred. The latter case is in agreement with a previous report (14).

Decreased rates of phosphorylation in chloroplasts and mitochondria at low temperatures probably contribute to decreased levels of high energy phosphates. Lyons and Raison (10) showed by Arrhenius plots that a transition occurs in the rate of mitochondrial activity of chilling-sensitive plants at temperatures between 9 and 12 C. Chilling-resistant plants showed no transition. They suggested that a phase change occurs in the membrane lipids of sensitive plants at low temperature, and they related this to the degree of unsaturation in the lipids (11). An Arrhenius plot of cotton seedling mitochondrial activity (Stewart and Guinn, in preparation) indicated that such a change also occurs in cotton.

However, if decreased levels of triphosphates at low temperatures were due only to decreased rates of phosphorylation, concomitant increases in diphosphates would be expected. In fact, mono- and diphosphates decreased with chilling with the exception of AMP in one experiment each with leaves and roots. The increase in AMP in these cases may be due to the large amounts of ADP and ATP hydrolyzed to AMP.

Table III gives the results of two experiments where the level of nucleosides was determined in control and chilled leaf tissue. These results indicate that chilling causes substantial increases in the nucleosides of cotton leaves. These increases in nucleosides probably are the result of dephosphorylation of the nucleotides when cotton plants are chilled.

The available data suggest that phosphatase activity in unhardened, chilled plants is relatively high compared to phosphorylating activity. Although phosphorylation is no doubt

Table III. *Nucleosides Extracted from Control and Chilled Cotton Leaves*

Experiment No.	Adenosine		Guanosine		Uridine + Cytidine ¹	
	1	2	1	2	1	2
<i>μmoles/g dry wt</i>						
Control	570	270	1450	860	3380	860
Chilled	730	880	2280	1420	3386	1130

¹ Uridine and cytidine were pooled because of poor separation. The extinction coefficient for uridine was used because cytidine was present in very small amounts.

affected, the overall decrease in nucleotides along with the increase in nucleosides indicates that hydrolytic processes are increased with chilling temperatures. The mechanisms involved in hardening condition the plant such that this hydrolytic action does not occur when the plant is chilled. Both the increased hydrolytic activity with chilling and the hardening process are probably associated with membrane structural changes.

LITERATURE CITED

- BROWN, E. G. 1962. The acid-soluble nucleotides of mature pea seeds. *Biochem. J.* 85: 633-642.
- CHANG, C. W. 1967. Effect of fluoride on nucleotides and ribonucleic acid in germinating corn seedling roots. *Plant Physiol.* 43: 669-674.
- CHERRY, J. H. AND R. H. HAGEMAN. 1960. Separation and identification of soluble nucleotides from etiolated corn seedlings as a function of growth. *Plant Physiol.* 35: 343-352.
- CIOTTI, M. AND N. O. KAPLAN. 1957. Procedures for determination of

- pyridine nucleotides. *In*: S. P. Colowick and N. O. Kaplan, eds. *Methods in Enzymology*, Vol. III. Academic Press, Inc., New York. pp. 890-899.
5. COHN, W. E. 1957. Methods of isolation and characterization of mono- and poly-nucleotides by ion exchange chromatography. *In*: S. P. Colowick and N. O. Kaplan, eds. *Methods in Enzymology*, Vol. III. Academic Press, Inc., New York. pp. 731-732.
 6. HURLBERT, R. B. 1957. Preparation of nucleoside diphosphates and triphosphates. *In*: S. P. Colowick and N. O. Kaplan, eds. *Methods in Enzymology*, Vol. III. Academic Press, Inc., New York. pp. 785-805.
 7. HURLBERT, R. B., H. SCHMITZ, A. F. BRUMM, AND V. R. POTTER. 1954. Nucleotide metabolism. II. Chromatographic separation of acid-soluble nucleotides. *J. Biol. Chem.* 209: 23-39.
 8. INGLE, J. A. 1962. A method for the improved separation of soluble nucleotides by ion-exchange chromatography. *Biochem. Biophys. Acta* 61: 147-149.
 9. KURAKSHI, S., N. ARAI, T. USHIJIMA, AND T. TAZAKI. 1968. Oxidized and reduced nicotinamide dinucleotide phosphate levels of plants hardened and unhardened against chilling injury. *Plant Physiol.* 43: 238-242.
 10. LYONS, J. M. AND J. K. RAISON. 1970. Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. *Plant Physiol.* 45: 386-389.
 11. LYONS, J. M., T. A. WHEATON, AND H. K. PRATT. 1964. Relationship between the physical nature of mitochondrial membranes and chilling sensitivity in plants. *Plant Physiol.* 39: 262-268.
 12. Pabst Laboratories, Division of Pabst Brewing Co., Circular OR-10, 1037 W. McKinley Ave., Milwaukee, Wis.
 13. ROBINSON, T. 1963. *The Organic Constituents of Higher Plants*. Burgess, Minneapolis.
 14. STEWART, J. MCD. AND G. GUINN. 1969. Chilling injury and changes in adenosine triphosphate of cotton seedlings. *Plant Physiol.* 44: 605-608.
 15. STREHLER, B. L. AND J. R. TOTTER. 1952. Firefly luminescence in the study of biological energy transfer systems. *Arch. Biochem. Biophys.* 40: 28-41.
 16. WEINSTEIN, L. H., D. C. McCUNE, J. F. MANCINI, AND P. VAN LEUKEN. 1969. Acid-soluble nucleotides of Pinto bean leaves at different stages of development. *Plant Physiol.* 44: 1499-1510.